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<b>(21) International Application Number:</b> PCT/US90/05294 <b>(22) International Filing Date:</b> 18 September 1990 (18.09.90) <b>(30) Priority data:</b> 410,647                      21 September 1989 (21.09.89) US <b>(71) Applicant:</b> MICRO VESICULAR SYSTEMS, INC. [US/ US]; 20 Cotton Road, Nashua, NH 03063 (US). <b>(72) Inventor:</b> WALLACH, Donald, F., H. ; 94 Dow Road, Hol- lis, NH 03049 (US). <b>(74) Agents:</b> LOREN, Ralph, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), BF (OAPI patent), BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (Euro- pean patent), CM (OAPI patent), DE (European pa- tent)*, DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (Eu- ropean patent), HU, IT (European patent), JP, LU (Eu- ropean patent), ML (OAPI patent), MR (OAPI patent), NL (European patent), NO, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI pa- tent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> HYBRID PAUCILAMELLAR LIPID VESICLES  <b>(57) Abstract</b>  Disclosed are hybrid paucilamellar lipid vesicles containing a phospho- or glycolipid and a nonionic, anionic or zwitteri- onic surfactant in the lipid bilayers. The paucilamellar vesicles may have either an aqueous or oil-filled central cavity. A method of manufacture for these vesicles is also disclosed. The paucilamellar lipid vesicles solve certain problems of cross-membrane transport, stability and cost, and may be used for transport of materials across membranes or skin, for diagnostic testing, or as markers or labels for visualization.		

\* See back of page

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HYBRID PAUCILAMELLAR LIPID VESICLESReference to Related Applications

This application is a continuation-in-part  
5 of United States Patent Application Serial No.  
157,571, filed March 3, 1988, entitled "Paucilamellar  
Lipid Vesicles," which was a continuation-in-part of  
United States Serial No. 025,525, filed March 13,  
1987, entitled "Method of Producing High Aqueous  
10 Volume Multilamellar Vesicles," now abandoned; United  
States Patent Application Serial No. 078,658, filed  
July 28, 1987, also entitled "Method of Producing  
High Aqueous Volume Multilamellar Vesicles," now  
United States Patent No. 4,855,090, issued August 8,  
15 1989; and United States Patent Application Serial No.  
124,824, filed November 25, 1987, entitled "Lipid  
Vesicles Formed of Surfactants and Steroids."

Background of the Invention

The present invention relates to the  
20 production of hybrid paucilamellar lipid vesicles.  
More particularly, the present invention concerns  
lipid vesicles which have phospholipids or  
glycolipids in addition to single-chain non-ionic,  
anionic, or zwitterionic surfactants as the major  
25 components of the walls (or lipid bilayers) of a  
paucilamellar lipid vesicle.

Lipid vesicles are substantially spherical  
structures made of materials having a high lipid  
content, e.g., surfactants or phospholipids. The

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lipids of these spherical vesicles are organized in the form of lipid bilayers. The lipid bilayers encapsulate an aqueous volume which is either interspersed between multiple onion-like shells of lipid bilayers (forming multilamellar lipid vesicles or "MLV") or the aqueous volume is contained within an amorphous central cavity. The most commonly known lipid vesicles having an amorphous central cavity filled with aqueous medium are the unilamellar lipid vesicles. Large unilamellar vesicles ("LUV") generally have a diameter greater than about 1  $\mu$  while small unilamellar lipid vesicles ("SUV") generally have a diameter of less than 0.2  $\mu$ . Lipid vesicles have a variety of uses including adjuvants or carriers for a broad spectrum of materials.

Although substantially all the investigation of lipid vesicles in recent years has centered on multilamellar and the two types of unilamellar lipid vesicles, a fourth type of lipid vesicle, the paucilamellar lipid vesicle ("PLV"), exists. See Callo and McGrath, Cryobiology 1985, 22(3), pp. 251-267. This lipid vesicle has barely been studied until recently and had only been manufactured with phospholipids surrounding an amorphous aqueous-filled volume. PLV's consist of about 2 to 10 peripheral bilayers surrounding a large, unstructured central cavity. In all PLV's described previous to United States Patent Application Serial No. 157,571, the disclosure of which is incorporated herein by reference, this central cavity was filled with an aqueous solution. The cited application first disclosed oil-filled vesicles.

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Each type of lipid vesicle appears to have certain uses for which it is best adapted. For example, MLV's have a higher lipid content than any of the other lipid vesicles, so to the extent that a lipid vesicle can encapsulate or carry a lipophilic material in the bilayers without degradation, MLV's have been deemed the most advantageous for carrying lipophilic materials. In contrast, the amount of water encapsulated in the aqueous shells between the lipid bilayers of the MLV's is much smaller than the water which can be encapsulated in the central cavity of LUV's, so LUV's have been considered advantageous in transport of aqueous material. However, LUV's, because of their single lipid bilayer structure, are not as physically durable as MLV's and are more subject to enzymatic degradation. SUV's have neither the lipid or aqueous volumes of the MLV's or LUV's but because of their small size have easiest access to cells in tissues.

PLV's, which can be considered a sub-class of the MLV's, possess features of both MLV's and LUV's. PLV's appear to have advantages as transport vehicles for many uses as compared with the other types of lipid vesicles. In particular, because of the large unstructured central cavity, PLV's are easily adaptable for transport of large quantities of aqueous- or oil-based materials. Moreover, the multiple lipid bilayers of the PLV's provides PLV's with additional physical strength and resistance to degradation as compared with the single lipid bilayer of the LUV's. As illustrated in the present application and the previously cited United States

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Patent Application Serial No. 157,571, the central cavity of the PLV's can be filled wholly or in part with an apolar oil or wax and then can be used as a vehicle for the transport or storage of hydrophobic materials. The amount of hydrophobic material which can be transported by the PLV's with an apolar core is much greater than can be transported by MLV's.

Conventional methods for producing multilamellar lipid vesicle start by dissolving the lipids, together with any lipophilic additives, in an organic solvent. The organic solvent is then removed by evaporation using heat or by passing a stream of an inert gas (e.g., nitrogen) over the dissolved lipids. The residue is then hydrated with an aqueous phase, generally containing electrolytes and additives such as hydrophilic biologically-active materials, to form multilamellar lipid membrane structures. In some variations, different types of particulate matter or structures have been used during the evaporation process to assist in the formation of the lipid residue. Changing the physical structure of the lipid residue can result in formation of better vesicles upon hydration. Two recent review publications, Gregoriadis, G., ed. Liposome Technology (CRC, Boca Raton, Fl.), Vols. 1-3 (1984), and Dousset and Douste-Blazy (in Les Liposomes, Puisieux and Delattre, Editors, Techniques et Documentation Lavoisier, Paris, pp.41-73 (1985)), summarize the methods which have been used to make MLV's.

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No matter how the MLV's or PLV's are formed, once made it is necessary to determine the effectiveness of the process. Two measurements commonly used to determine the effectiveness of encapsulation of materials in lipid vesicles are the encapsulated mass and captured volume. The encapsulated mass is the mass of the substance encapsulated per unit mass of the lipid and is often given as a percentage. The captured volume is defined as the amount of the aqueous phase trapped inside the vesicle divided by the amount of lipid in the vesicle structure, normally given in ml liquid/g lipid.

Phospholipid vesicles, while mimicking membrane structure because of similarity of materials with naturally occurring membranes, have a number of problems. First, isolated phospholipids are subject to degradation by a large variety of enzymes. Second, the most easily available phospholipids are those from natural sources, e.g., egg yolk lecithin, which contain polyunsaturated acyl chains that are subject to autocatalyzed peroxidation. When peroxidation occurs, the lipid structure breaks down, causing fracture of the lipid vesicle and premature release of any encapsulated material. While hydrogenation may be used to saturate the chains, it is an expensive process which raises the already high cost of the phospholipid starting materials, as well as changing the vesicle stability.

Because of these problems with using plain phospholipids, certain companies, primarily L'Oreal

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and Micro Vesicular Systems, have been using non-ionic surfactants to form the structure of vesicles. L'Oreal uses primarily polyglycols, e.g., see United States Patents Serial Nos. 4,772,471 and 5 4,217,344, while Micro Vesicular Systems has been using primarily polyoxyethylene fatty acid ethers and esters (see United States Patent Application Serial No. 157,571 and United States Patent No. 4,855,090). The L'Oreal vesicles appear to be classic MLV's while 10 the Micro Vesicular Systems vesicles are primarily PLV's.

For certain uses, e.g., transportation of vesicles through membranes or permeation of the skin, 15 the presence of a small amount of phospholipid and/or glycolipid to the bilayer structure of the vesicles may be important. A problem with using the phospholipids or glycolipids in conjunction with many synthetic surfactants is that most of the surfactants 20 have a non-ionic head group linked to a single hydrophobic chain while most phospholipids and glycolipids have two hydrophobic chains linked to an ionic head group. Use of both single and multiple chain molecules in the structure of vesicle walls may 25 lead to problems in the packing of the lipids which form the lipid bilayers. Under most circumstances, one would expect that any attempt to form a stable vesicle by blending single and multiple chain lipids, particularly when one is a non-ionic lipid while the 30 other is an ionic or zwitterionic lipid, would be difficult at best. In addition, the phospholipids are still subject to phospholipases after vesicle formation. However, the hybrid vesicles are exactly



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what is needed to solve certain problems of cross-membrane transport, stability and cost.

Accordingly, an object of the invention is to provide stable hybrid lipid vesicles having a  
5 non-ionic, zwitterionic, or anionic surfactant and a phospholipid or glycolipid in the lipid bilayers of the vesicles.

A further object of the invention is to provide stable hybrid paucilamellar lipid vesicles  
10 encapsulating a water-immiscible material within the central amorphous cavities of the vesicles.

Another object of the invention is to provide a method of manufacture of hybrid vesicles.

A still further object of the invention is  
15 to provide a vehicle for the transport of oil-soluble or water-soluble materials into the skin.

These and other objects and features of the invention will be apparent from the following description.

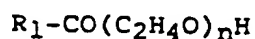
20 Summary of the Invention

The present invention features hybrid  
paucilamellar lipid vesicles having phospholipids or glycolipids in addition to single-chain non-ionic, anionic, or zwitterionic non-phospholipids in the  
25 lipid bilayers. These hybrid vesicles are

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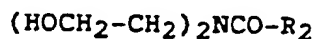
particularly useful for transport of oil-soluble or water-soluble material into the skin.

The hybrid paucilamellar lipid vesicles having phospholipids or glycolipids in addition to 5 non-ionic or zwitterionic surfactants in their lipid bilayers consist of 2-10 lipid bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. The lipid 10 bilayers have about 0-30% phospholipids and/or glycolipid and 0-75% single-chain non-ionic, anionic, or zwitterionic surfactant, preferably with other materials such as 0-25% of a sterol and 0-5% of a charge-producing agent. The preferred non-ionic 15 surfactants are selected from the group consisting of polyoxyethylene fatty ethers having the formula



where  $R_1$  is lauric, myristic, or palmitic acid or their derivatives, single or double 20 unsaturated octadecyl acids or their derivatives, or double unsaturated eicodienoic acids or their derivatives and  $n$  ranges from 2-4;

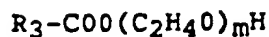
diethanolamides having the formula



25 where  $R_2$  is caprylic, lauric, myristic, palmitic, stearic, or linoleic acid or their derivatives;

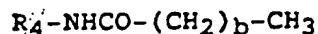
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polyoxyethylene fatty acid esters having the  
formula



where  $R_3$  is lauric, myristic, palmitic,  
5 stearic, or oleic acids or their derivatives, double  
unsaturated octadecyl acids or their derivatives, or  
double unsaturated eicodienoic acids or their  
derivatives and  $m$  ranges from 2-4;

long chain acyl hexosamides having the  
10 formula



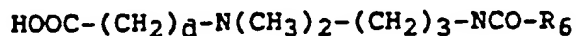
where  $b$  ranges from 10-18 and  $R_4$  is a sugar  
molecule selected from a group consisting of  
glucosamine, galactosamine, and N-methylglucamine;

15 long chain acyl amino acid amides having the  
formula



where  $c$  ranges from 10-18 and  $R_5$  is an amino  
acid side chain;

20 long chain acyl amides having the formula



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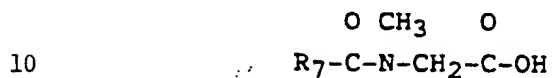
where  $R_6$  is an acyl chain having 12-20 carbons and not more than two unsaturations, and d ranges from 1-3;

polyoxyethylene (20) sorbitan mono- or trioleate;

5 polyoxyethylene glyceryl monostearate with 1-10 polyoxyethylene groups;

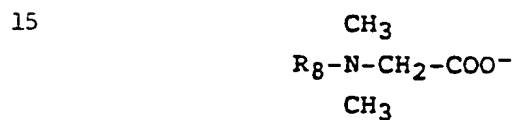
glycerol monostearate;

sarcosinamides having the formula

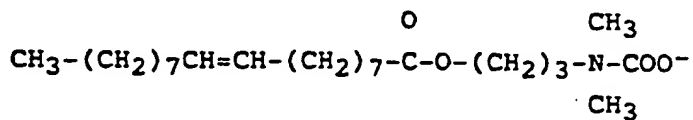


where  $R_7$  is selected from the group consisting of single-chain carbonyl derivatives of  $C_{12}$ - $C_{20}$  fatty acids; and

betaines having the formula



where  $R_8$  is selected from the group consisting of long-chain fatty acid esters, most  
20 preferably oleoyl propyl betaine having the formula



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Phospholipids and/or glycolipids particularly useful in the invention include phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, inositolphosphatides, sphingomyelins, ceramides, cerebrosides, gangliosides, sulfatides, and mixtures and derivatives thereof. Phospholipids or glycolipids with saturated hydrocarbon chain length greater than sixteen are not as useful as smaller chain or unsaturated chain molecules in the invention because they lack some chain fluidity. If a charge-producing agent is used, preferred charge-producing agents include dicetyl phosphate, quaternary ammonium salts, cetyl sulfate, sarcosinamides, phosphatidic acid, phosphatidyl serine, and fatty acids such as oleic acid or palmitic acid.

The preferred hybrid paucilamellar vesicles of the invention have a central cavity carrying either water-soluble materials or water-immiscible oily-solution, preferably selected from a group consisting of oils, waxes, natural and synthetic triglycerides, acyl ethers, petroleum derivatives and their analogues and derivatives, encapsulated within the central amorphous cavity. This type of water-immiscible material can act as a carrier for materials which are not soluble in an aqueous phase. In addition, it may also be used for materials which are not dissolvable or soluble in the lipids which form the bilayers. If the water-immiscible oil, such as a peanut oil, tristearin or mineral oil is used, the amount of non-ionic or zwitterionic surfactant which is needed to form stable vesicles is

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decreased. In fact, under certain circumstances, oil-filled paucilamellar vesicles may be formed using the methods of the invention without the addition of any non-ionic or zwitterionic surfactant. However, 5 the most stable hybrid vesicles appear to be formed with 10-30% phospholipid and/or glycolipid, 5-20% of a sterol which is cholesterol, about 1% of a charge-producing agent such as oleic acid, and the remainder constituting the surfactant.

10 The invention further features a method of producing the hybrid vesicles of the invention. The phospholipid and/or glycolipid and non-ionic or zwitterionic surfactant are blended, with heating if necessary, until a homogeneous lipid layer is 15 formed. If a water-immiscible oil is to be encapsulated, it is blended in the already formed lipid phase, forming a lipophilic phase. If any oil-soluble or oil-suspendable materials are to be encapsulated within the paucilamellar vesicles, they 20 are first dispersed in the oil. The term "dispersed" as used herein includes dissolution or forming a suspension or colloid to yield a flowable phase. If no oil is used, the lipid phase is the lipophilic phase.

25 Once a lipophilic phase is made, it is blended with an aqueous phase under shear mixing conditions to form the vesicles. "Shear mixing" is defined as the mixing of the lipophilic phase with the aqueous phase under turbulent or shear conditions 30 which provide adequate mixing to hydrate the lipid and form lipid vesicles. Shear mixing achieved by

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liquid shear which is substantially equivalent to a relative flow rate for the combined phases of about 5-30 m/s to a 1 mm orifice. The use of shear mixing conditions disrupts any lamellae which may form so  
5 that the vesicles are formed without the formation of a separable lamellar phase.

The same materials which have been previously described for the vesicles are useful in the methods of the invention. Briefly, the formed  
10 lipophilic phase is shear mixed with an excess of aqueous, e.g., 10:1:aqueous:lipid, and the resulting vesicles, which form in under a second, are then separated and can be used any of a variety of other uses.

15 The following description will further explain the invention.

#### Detailed Description of the Invention

The hybrid vesicles of the invention can be used for a variety of purposes, including the  
20 function of a carrier for transport of materials across membranes or skin that would otherwise not be transportable. In fact, the vesicles of the invention could be used for any purpose where lipid vesicles such as liposomes are now being used or  
25 contemplated. These lipid vesicles are characterized by 2-10 lipid bilayers or shells with small aqueous volumes separating each substantially spherical lipid shell. The innermost lipid bilayer surrounds a large, substantially amorphous central cavity which

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may be filled with either an aqueous solution or a water-immiscible oil. This central cavity acts as a "cargo hold," allowing delivery of a variety of materials to the desired location.

- 5           For certain uses, the incorporation of a charge producing amphiphile, yielding a net positive or negative charge to the lipid vesicles, is helpful. The preferred negative charge producing materials are carboxylic acids such as oleic and
- 10 palmitic acids, dicetyl phosphate, cetyl sulphate, sacrosinamides, phosphatidic acid, phosphatidyl serine, and mixtures thereof. In order to provide a net positive charge to the vesicles, long chain
- 15 amines, e.g., stearyl amines or oleyl amines, cationic local anaesthetics such as lidocaine, long chain pyridinium compounds, e.g., cetyl pyridinium chloride, quaternary ammonium compounds, or mixtures of these can be used.

- The vesicles may also include targeting
- 20 molecules, either hydrophilic or amphiphilic, which can be used to direct the vesicles to a particular target in order to allow release of the material encapsulated in the vesicle at a specified biological location. If hydrophilic targeting molecules are
- 25 used, they can be coupled directly or via a spacer to a residue of the polar portion of the surfactant, or they can be coupled, using state of the art procedures, to molecules such as palmitic acid, long chain amines, or phosphatidyl ethanolamine. If
- 30 spacers are used, the targeting molecules can be interdigitated into the hydrophilic core of the



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bilayer membrane via the acyl chains of these compounds. Preferred hydrophilic targeting molecules include monoclonal antibodies, other immunoglobulins, lectins, and peptide hormones.

5           For certain uses, targeting molecules which were linked to the lipid bilayers themselves, through the use of a sulfhydryl bond, are preferred. United States Patent Application Serial No. 320,944 describes a linkage of this type which yields high  
10 efficiency targeting. Molecules containing sulfhydryl groups are incorporated into the structure of the bilayers and a bifunctional cross-linking reagent is used to link the targeting molecule to the bilayers.

15           In addition to hydrophilic targeting molecules, it is also possible to use amphiphilic targeting molecules. Amphiphilic targeting molecules are normally not chemically coupled to the surfactant molecules but rather interact with the lipophilic or  
20 hydrophobic portions of the molecules constituting the bilayer lamellae of the lipid vesicles. Preferred amphiphilic targeting molecules are neutral glycolipids, galactocerebrosides (e.g., for hepatic galactosyl receptors), or charged glycolipids such as  
25 gangliosides.

Vesicles made using the methods of the present invention can be used in diagnostic testing, e.g., agglutination testing of immunological systems. The vesicles can also be used as markers or  
30 labels for visualization, e.g., swelling or shrinking

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in the presence of an immune reaction, or for radiography or NMR.

Hydrophilic materials which can be encapsulated include minerals such as titanium  
5 dioxide and silicas, viruses, macromolecules, immunological adjuvants such as muramyl dipeptide, peptide hormones such as insulin, calcitonin and glucagon, hypothalamic peptides, pituitary hormones, growth factors such as angiogenic, epithelial and  
10 epidermal growth factors, lymphokines such as interleukin-2 and interferon, blood proteins such as hemoglobin and Factor VIII, water-soluble plant hormones and pesticides, radionucleotides, contrast materials for radiological and NMR diagnosis, cancer  
15 cytostatics, and antibiotics. Examples of lipophilic materials which can be encapsulated include steroid hormones, pheromones, porphyrins, organic pesticides, fungicides, insect repellents, lipophilic vitamins and derivatives, alkyds, epoxy polyurethanes,  
20 fluorocarbons, and related resins. Oil based materials include an exclusive listing of additional lipophilic materials and materials which form colloids or suspensions in oil. A more complete listing of the types of pharmaceuticals that could be  
25 encapsulated in lipid vesicles is included in Gregoriadis, G., ed. Liposome Technology (CRC, Boca Raton, Fl.), Vols. 1-3 (1984).

The paucilamellar lipid vesicles can be made by a variety of devices which provides sufficiently  
30 high shear for shear mixing. There are a large variety of these devices available on the market

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including a microfluidizer such as is made by  
Biotechnology Development Corporation, a  
"French"-type press, or some other device which  
provides a high enough shear force and the ability to  
5 handle heated, semiviscous lipids. If a very high  
shear device is used, it may be possible to  
microemulsify powdered lipids, under pressure, at a  
temperature below their normal melting points and  
still form the lipid vesicles of the present  
10 invention.

A device which is particularly useful for  
making the lipid vesicles of the present invention  
has been developed by Micro Vesicular Systems, Inc.,  
Vineland, New Jersey and is further described in  
15 United States Patent Application Serial No. 163,806,  
filed May 3, 1988. Briefly, this device has a  
substantially cylindrical mixing chamber with at  
least one tangentially located inlet orifice. One or  
more orifices lead to a reservoir for the lipophilic  
20 phase, mixed with an oil phase if lipid-core PLV's  
are to be formed, and at least one of the other  
orifices is attached to a reservoir for the aqueous  
phase. The different phases are driven into the  
cylindrical chamber through pumps, e.g., positive  
25 displacement pumps, and intersect in such a manner as  
to form a turbulent flow within the chamber. The  
paucilamellar lipid vesicles form rapidly, e.g., less  
than 1 second, and are removed from the chamber  
through an axially located discharge orifice. In a  
30 preferred embodiment, there are four tangentially  
located inlet orifices and the lipid and aqueous  
phases are drawn from reservoirs, through positive

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displacement pumps, to alternating orifices. The fluid stream through the tangential orifices is guided in a spiral flow path from each inlet or injection orifice to the discharge orifice. The flow  
5 paths are controlled by the orientation or placement of the inlet or injection orifices so as to create a mixing zone by the intersection of the streams of liquid. The pump speeds, as well as the orifice and feed line diameters, are selected to achieve proper  
10 shear mixing for lipid vesicle formation. As noted, in most circumstances, turbulent flow is selected to provide adequate mixing.

For small scale, e.g., experimental uses, shear mixing may be carried out using merely a series  
15 of syringes and a stopcock joining them. This "syringe method" uses one syringe containing the lipophilic phase, heated if necessary for flowability, which is then linked, via a stopcock, to a second, larger syringe containing an excess of an  
20 aqueous phase. The lipid and aqueous phase are then blended rapidly through the stopcock for a short time, e.g., normally less than a minute. This blending causes sufficient shearing to form the paucilamellar vesicles of the invention without the  
25 formation of an intermediate or separate lamellar phase. In fact, the formation of a separable lamellar phase would so clog or disrupt the flow as to make this method impossible to use.

When the water-immiscible oil is used, the  
30 oil displaces a portion of the aqueous phase as the vesicles are formed. The oil stabilizes the

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vesicles, leading to high fracture strength and longer term stability than vesicles made without oil. Although it is not necessary for understanding the invention, it is theorized that a very small  
5 amount of the surfactant acts as a stabilizing agent, stabilizing the boundary between the aqueous volume and the oil volume, allowing the oil droplet to form. In fact, the oil-filled vesicles are so stable that paucilamellar oil-filled vesicles can be formed  
10 from phospholipids using the methods of the invention without the addition of any non-ionic or zwitterionic surfactant, while aqueous-filled vesicles cannot be formed using the same materials and methods.

The following Examples will more clearly  
15 illustrate and delineate the scope of the present invention.

Example 1.

In this invention, egg yolk  
phosphatidylcholine (Lipoid 100, Lipoid ViCt,  
20 Ludwigshafen, GFR), was used in conjunction with a polyoxyethylene ether surfactant to form stable aqueous-based vesicles. These vesicles show high lipid uptake per/g of lipid and small size.

Table I lists the ingredients used to make  
25 the vesicles of this Example.

- 20 -

TABLE 1.

Egg Yolk Phosphatidylcholine	1.00 g
Polyoxyethylene-2 Cetyl Ether (Brij 52)	1.00 g
Cholesterol	0.25 g
5 Oleic Acid	0.02 g

Briefly, approximately 1 g of egg yolk phosphatidylcholine was blended with 1 g of polyoxyethylene-2 cetyl ether (Brij 52, ICI Americas, Inc.), 0.25 g cholesterol (Sigma Chemical Co.) and  
10 0.02 g oleic acid (J. T. Baker) as a charge-producing agent at approximately 65°C. A homogeneous lipid phase was obtained. One ml of the lipid phase was then sheared mixed with 9 ml of phosphate buffered saline for approximately 2 minutes using a syringe  
15 method as described below.

The lipophilic phase is placed in a 10 ml syringe and is attached through a stopcock having about a 1 mm orifice to a 25 ml syringe which contains the aqueous phase, phosphate buffered  
20 saline. After the injection of the lipid phase into the aqueous phase, a second 25 ml syringe replaces the 10 ml syringe and the solution is rapidly forced through the stopcock from one syringe to the other. The resulting vesicles form in less than 1 minute.

25 The milky suspension which was obtained by the syringe method was combined with 20% dextran in saline at a centrifuge of 3000 rpm's for 15 minutes in a Beckman GP centrifuge. A liposomal layer separated at the top of the centrifuge tube.

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Microscopic examination showed spherical paucilamellar lipid vesicles which were not distinguishable from vesicles which did not have the phosphatidylcholine. The mean particle diameter was approximately 0.171  $\mu$  and the volume uptake was approximately 7.2 ml of phosphate buffered saline/g lipid.

Example 2.

This Example illustrates the oil-based paucilamellar vesicles of the invention. A lipid phase identical to that described in Example 1 was manufactured. One ml of that lipid phase was then blended with 1 ml of mineral oil (Drakeol 19), forming a lipophilic phase. The resulting 2 ml of the lipophilic phase was then blended with 9 ml of phosphate buffered saline using the syringe technique as described above.

Again, a milky solution is obtained which upon dextran centrifugation yields oil-filled paucilamellar vesicles. All of the oil is encapsulated within the vesicles, which also have a volume uptake of approximately 3 ml of the phosphate buffered saline/g lipid (excluding the mineral oil). The mean particle diameter was approximately 0.654  $\mu$ .

Example 3.

This Example illustrates that the methods of the invention may be used to form phospholipid

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paucilamellar oil-filled vesicles. Table 2 gives the ingredients used.

TABLE 2.

	Egg Yolk Phosphatidylcholine	1.00 g
5	Cholesterol	0.20 g
	Oleic Acid	0.02 g
	Mineral Oil (Drakeol 19)	1 ml

One g of egg yolk phosphatidylcholine was blended with 0.2 g cholesterol and 0.02 g oleic acid by heating to 65°. This lipid phase was then blended with 1 g mineral oil (Drakeol 19) to form a lipophilic phase. Approximately 2 ml of lipophilic phase was then blended, using the syringe technique as previously described, with 4 ml of phosphate buffered saline. The resulting milky suspension was separated on a 20% dextran and saline gradient using centrifugation of 3,000 rpm for 15 minutes. A 3 ml liposomal layer separated on top of the centrifuge tube.

Microscopic examination showed spherical, oil-filled paucilamellar lipid vesicles. The aqueous volume uptake was approximately 3 ml/g phospholipid.

Example 4.

This Example and the following Examples (Examples 5-7) illustrate the use of a mixed phospholipid/glycolipid as part of the structure of the paucilamellar vesicles of the invention. Table 3



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illustrates the materials used to form the lipid bilayers in this Example.

TABLE 3.

Type VIII Brain Extract	0.76 g
5 Polyoxyethylene-5 Oleyl Ether	1.99 g
Cholesterol	0.58 g
Oleic Acid	0.08 g

The type VIII brain extract, which is 30% sphingomyelin (a phospholipid), 30% cerebroside (a glycolipid), 10% sulfatide, and the balance other brain lipids is blended with polyoxyethylene-5 oleyl ether, cholesterol and oleic acid at approximately 65°C. to form a lipid phase. Approximately 1 ml of this lipid phase was mixed with 9 ml of phosphate  
15 buffered saline using the previously described syringe technique. Upon dextran centrifugation, spherical lipid vesicles encapsulating an aqueous phase could be seen under a microscope. The mean particle diameter was approximately 0.21  $\mu$  and the  
20 volume uptake was approximately 2.7 ml saline/g lipid.

Example 5.

In this Example, the mixed brain extract of Example 4 was used to make oil-centered vesicles. The lipid phase was made as described in Example 4  
25 and 1 ml of the lipid phase was combined with an equal volume of peanut oil. The resulting mixture was made into vesicles using the same syringe technique described in Example 4. After dextran

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centrifugation, vesicles were separated which had a mean diameter of 1.66 microns and a volume uptake of 3.5 ml liquid/g lipid.

Surprisingly, use of mineral oil in place of the peanut oil would not produce vesicles.

Example 6.

In this Example and the following Example, the same brain extract as was used in Examples 4 and 5 is used to make vesicles, except an entirely different type of surfactant, diethanolamine linoleamide, was added.

Table 4 shows the lipids used in the manufacture of the lipid phase of this Example.

TABLE 4.

15	Type VIII Brain Extract	0.76 g
	Diethanolamine Linoleamide	1.78 g
	Cholesterol	0.85 g
	Oleic Acid	0.10 g

Approximately 0.76 g of the type VIII brain extract was blended with 1.78 g of diethanolamine linoleamide, 0.85 g cholesterol and 0.10 g oleic acid at 65°C. One ml of this lipid phase was mixed, using the syringe technique, with 8 ml of phosphate buffered saline. After dextran centrifugation, lipid vesicles were observed, showing a mean particle

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diameter of approximately 0.263  $\mu$ . The volume uptake was 5 ml of saline/g lipid.

Example 7.

In this Example, the same lipid phase was  
5 used as in Example 6 except oil-centered vesicles  
were made. After the formation of the lipid phase,  
1 ml of the lipid phase was blended with 1 ml peanut  
oil before shear mixing using the syringe technique  
with 8 ml of the phosphate buffered saline. Upon  
10 separation, spherical vesicles with the mean particle  
diameter of approximately 0.323  $\mu$  were observed. The  
volume uptake was approximately 4.5 ml/g lipid.

As with Example 6, mineral oil would not  
form vesicles.

15 As can be seen from the results of these  
Examples, paucilamellar lipid vesicles having high  
water or oil uptake can be formed with the materials  
and methods of the present invention. Other testing  
has shown that if different methods are used, e.g.,  
20 the Bangham method for manufacture of lipid vesicles,  
paucilamellar lipid vesicles are not formed using the  
same materials but rather classic multilamellar lipid  
vesicles are formed. These MLV's yield a much lower  
water uptake as compared with PLV's and they exhibit  
25 substantially no oil uptake.

The foregoing description is illustrative  
only and those skilled in the art may find other

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materials and methods which accomplish the same results. Such other materials and methods are included within the following claims.

What is claimed is:

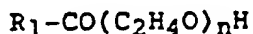
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1. Hybrid paucilamellar lipid vesicles having a first lipid selected from a group consisting of phospholipids, glycolipids, and mixtures thereof, and a second lipid selected from the group consisting of  
5 non-ionic surfactants, zwitterionic surfactants, anionic surfactants, and mixtures thereof, as structural materials in lipid bilayers, said vesicle containing 2-10 lipid bilayers in the form of concentric substantially spherical shells separating  
10 aqueous layers, the innermost of said lipid bilayers enclosing a substantially amorphous central cavity, said vesicle bilayers comprising:

0-30% said first lipid; and

0-75% second lipid, said second lipid being  
15 selected from the group consisting of

polyoxyethylene fatty acid ethers having the formula



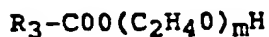
where  $R_1$  is lauric, myristic, or palmitic  
20 acid or their derivatives, single or double unsaturated octadecyl acids or their derivatives, or double unsaturated eicodienoic acids or their derivatives, and  $n$  ranges from 2-10; and

diethanolamides having the formula



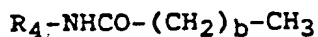
- 28 -

where  $R_2$  is caprylic, lauric, myristic, stearic, or linoleic acid or their derivatives;



where  $R_3$  is lauric, myristic, palmitic, stearic, or oleic acids or their derivatives, double unsaturated octadecyl acids or their derivatives, or double unsaturated eicodienoic acids or their derivatives and  $m$  ranges from 2-4;

long chain acyl hexosamides having the  
10 formula



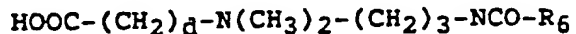
where  $b$  ranges from 10-18 and  $R_4$  is a sugar molecule selected from a group consisting of glucosamine, galactosamine, and N-methylglucamine;

15 long chain acyl amino acid amides having the formula



where  $c$  ranges from 10-18 and  $R_5$  is an amino acid side chain;

20 long chain acyl amides having the formula



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where  $R_6$  is an acyl chain having 12-20 carbons and not more than two unsaturations, and d ranges from 1-3;

polyoxyethylene (20) sorbitan mono- or trioleate;

5 polyoxyethylene glyceryl monostearate with 1-10 polyoxyethylene groups;

glycerol monostearate;

sarcosinamides having the formula



where  $R_7$  is selected from the group consisting of single-chain carbonyl derivatives of  $C_{12}$ - $C_{20}$  fatty acids; and

betaines having the formula



where  $R_8$  is selected from the group consisting of long-chain fatty acid esters.

20 2. The hybrid vesicles of claim 1 wherein said lipid bilayers further comprise a sterol or a derivative or chemical analogue thereof.

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3. The hybrid vesicles of claim 2 wherein said sterol is selected from the group consisting of cholesterol, hydrocortisone, and derivatives and chemical analogues thereof.

5 4. The hybrid vesicles of claim 2 wherein said lipid bilayers further comprise a charge-producing agent.

5. The hybrid vesicles of claim 4 wherein said charge-producing agent is selected from the group  
10 consisting of carboxylic acids, dicetyl phosphate, cetyl sulfate, phosphatidic acid, phosphatidyl serine, quaternary ammonium salts, cationic local anaesthetics, and mixtures and derivatives thereof.

6. The hybrid vesicles of claim 1 wherein said  
15 first lipid is selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, inositolphosphatides, sphingomyelins, ceramides, cerebrosides, gangliosides, sulfatides, and derivatives and  
20 chemical analogues thereof.

7. The hybrid vesicles of claim 1 wherein said vesicles comprises oil-filled paucilamellar lipid vesicles.

8. The hybrid vesicles of claim 7 wherein said  
25 amorphous central cavity of said oil-filled paucilamellar vesicles comprises a water-immiscible oil.



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9. The hybrid vesicles of claim 8 wherein said water-immiscible oil is selected from the group consisting of oils, waxes, natural and synthetic triglycerides, acyl ethers, petroleum derivatives, 5 and their derivatives and chemical analogues.

10. A method of making hybrid paucilamellar lipid vesicles comprising the steps of:

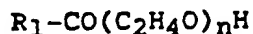
forming a lipid phase by blending a first lipid selected from the group consisting of 10 phospholipids, glycolipids, and mixtures thereof, a second lipid selected from the group consisting of non-ionic surfactants, zwitterionic surfactants, anionic surfactants, and mixtures thereof, and any other lipophilic molecules to be incorporated into 15 the lipid bilayers of said vesicles;

forming an aqueous phase by blending an aqueous-based solution and any aqueous soluble materials to be incorporated into said vesicles; and

shear mixing said lipid phase and said 20 aqueous phase to form said hybrid vesicles without the formation of a separable lamellar phase.

11. The method of claim 10 wherein said second lipids are selected from the group consisting of

polyoxyethylene fatty acid ethers having the 25 formula



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where  $R_1$  is lauric, myristic, or palmitic acid or their derivatives, single or double unsaturated octadecyl acids or their derivatives, or double unsaturated eicodienoic acids or their derivatives, and  $n$  ranges from 2-10; and

diethanolamides having the formula

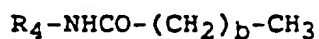


where  $R_2$  is caprylic, lauric, myristic, stearic, or linoleic acid or their derivatives.

10  $R_3\text{-COO}(\text{C}_2\text{H}_4\text{O})_m\text{H}$

where  $R_3$  is lauric, myristic, palmitic, stearic, or oleic acids or their derivatives, double unsaturated octadecyl acids or their derivatives, or double unsaturated eicodienoic acids or their derivatives and  $m$  ranges from 2-4;

long chain acyl hexosamides having the formula



where  $b$  ranges from 10-18 and  $R_4$  is a sugar molecule selected from a group consisting of glucosamine, galactosamine, and N-methylglucamine;

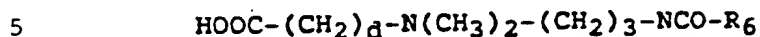
long chain acyl amino acid amides having the formula

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where c ranges from 10-18 and R<sub>5</sub> is an amino acid side chain;

long chain acyl amides having the formula



where R<sub>6</sub> is an acyl chain having 12-20 carbons and not more than two unsaturations, and d ranges from 1-3;

polyoxyethylene (20) sorbitan mono- or trioleate;

10 polyoxyethylene glyceryl monostearate with 1-10 polyoxyethylene groups;

glycerol monostearate;

sarcosinamides having the formula



where R<sub>7</sub> is selected from the group consisting of single-chain carbonyl derivatives of C<sub>12</sub>-C<sub>20</sub> fatty acids; and

betaines having the formula



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where R<sub>8</sub> is selected from the group consisting of long-chain fatty acid esters.

12. The method of claim 11 wherein said lipid phase further comprises a sterol.

5 13. The method of claim 12 wherein said sterol is selected from the group consisting of cholesterol, hydrocortisone, and derivatives and chemical analogues thereof.

14. The method of claim 11 wherein said lipid  
10 phase further comprises a charge-producing agent.

15. The method of claim 14 wherein said charge-producing agent is selected from the group consisting of carboxylic acids, dicetyl phosphate, cetyl sulfate, phosphatidic acid, phosphatidyl  
15 serine, quaternary ammonium salts, and mixtures and derivatives thereof.

16. The method of claim 11 wherein said first lipid is selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines,  
20 phosphatidylserines, inositolphosphatides, sphingomyelins, ceramides, cerebrosides, gangliosides, sulfatides, and derivatives and chemical analogues thereof.

17. The method of claim 11 wherein said hybrid  
25 paucilamellar vesicles are oil-filled hybrid paucilamellar vesicles, said method comprising the further steps of:

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forming an oily phase by blending any oil dispersible material to be encapsulated in said vesicles in a water-immiscible oily solution; and

blending said lipid phase and said oily phase to form a lipophilic phase both shear mixing with said aqueous phase to form said vesicles.

18. The method of claim 17 wherein said water-immiscible oily solution is selected from the group consisting of oils, waxes, natural and  
10 synthetic triglycerides, acyl ethers, petroleum derivatives, and their derivatives and chemical analogues.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/05294

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC: INT. Cl. (5): A61K 9/127; 37/22; B01J 13/02 U.S. Cl. 264/4.1; 424/450; 428/402.2; 436/829		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched †		
Classification System	Classification Symbols	
U.S. Cl.	264/4.1; 424/450; 428/402.2; 436/829	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, † with indication, where appropriate, of the relevant passages ‡	Relevant to Claim No. ‡
X	US, A, 4,217,344 (Vanlerberghe et al) 12 August 1980 See examples 1,2,13,19,23 & 25; and Col.4, lines 22,27; and Col.4, line 56 - Col. 5, line 44	1-6,10-16
X	US, A, 4,247,411 (Vanlerberghe et al) 27 January 1981 See examples 2,3,6&8; and Col.3, line 25 - Col.4, Line 41.	1-6,10-16
X	US, A, 4,448,765 (ASH et al) 15 May 1984 See examples 1-6; and Col.4, line 62 - Col.5, Line 40.	1-6,10-16
X	"Les Niosomes" (Handjani-Vila et al) 1985, pages 297-313. See especially pages 302-309.	1-6, 10-16
X	US,A, 4,911,928 (Wallach) 27 March 1990 See patent claims 1-12.	7-9,17,18
X	US, A, 4,942,038 (Wallach) 17 July 1990 See patent claims 1-4, 18 and 19.	7-9
<p>* Special categories of cited documents: †</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
17 December 1990		29 JAN 1991
International Searching Authority		Signature of Authorized Person
ISA/US		CHUYEN NGOC-HO INTERNATIONAL DIVISION Richard D. Lovering